Amendments to the Specification

Please replace the paragraph on page 1, line 14, to page 2, line 2, with the following amended paragraph:

This application is related to corresponding International PCT application No. attorney docket No. 37851-923PC, entitled RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING. This application also is related to U.S. application Serial No. ~~attorney docket number 37851-922~~ 10/658,834, entitled "RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, ENCODING NUCLEIC ACID MOLECULES AND RELATED APPLICATIONS," filed the same day herewith; to U.S. Provisional Application Serial No. 60/457,135, entitled "RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, ENCODING NUCLEIC ACID MOLECULES AND RELATED APPLICATIONS;" filed March 21, 2003, and to U.S. Provisional Application Serial No. 60/409,898, entitled "RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, ENCODING NUCLEIC ACID MOLECULES AND RELATED APPLICATIONS," filed September 9, 2002, each to Rene Gantier, Thierry Guyon, Manuel Vega and Lila Drittanti. This application also is related to co-pending U.S. application Serial No. 10/022,249, filed December 17, 2001, entitled "HIGH THROUGHPUT DIRECTED EVOLUTION BY RATIONAL MUTAGENESIS," to Manuel Vega and Lila Drittanti.

Please replace the paragraph on page 4, lines 4-15, with the following amended paragraph:

Target loci in a protein for ~~modificiation~~modification are selected based on properties of the target ~~polyeptide~~polypeptide, including *i*) the particular protein properties to be evolved, *ii*) the protein's amino acid sequence, and *iii*) the known properties of the individual amino acids, a number of target amino acid positions along the protein sequence are selected *in silico* for replacement. The

target loci (amino acid positions) along the protein sequence selected *in silico* for modification, typically replacement, are referred to as "is-HIT target positions." The number of is-HIT target positions is generally selected to be as large as possible such that all reasonably possible target positions for the particular feature being evolved are identified and included. In particular embodiments less than all are identified.

Please replace the paragraph on page 5, lines 16-29, with the following amended paragraph:

Figure 1(A) shows a schematic of the initial step in the methods provided herein for 2D-scanning. Once the protein feature(s) to be optimized is (are) selected (indicated as "?"), diverse sources of information or previous knowledge (i.e., protein primary, secondary or tertiary structures, literature, patents) are exploited to determine those amino acid positions that may be amenable to improved protein fitness by replacement with a different amino acid. This step utilizes protein analysis "*in silico*." All possible candidate positions that might be involved in the feature being evolved are referred to herein as "*in silico* HITs" ("is-HITs"). The collection (or library) of all is-HITs identified during this step represents the first dimension (target residue position) of the two-dimensional scanning methods provided herein. The first dimension is restricted because only ~~aminoacids~~ amino acids along the protein sequence that are the is-HITs.

Please replace the paragraph on page 6, line 22, to page 7, line 2, with the following amended paragraph:

Figure 3(A) shows a further step in the methods provided herein for rational evolution of peptides and proteins. Following identification of LEADs, a new collection of mutant molecules is obtained by combination of any two or more of the mutations present in the LEAD molecules. The collection of new mutant molecules is generated, tested and phenotypically characterized such as

in the [[the]] one-by-one in addressable arrays exemplified in the Figure. Each individual mutant in the collection is designed and produced as the single product of an independent mutagenesis reaction. Mutant molecules are such that each molecule contains a variable number and type of LEAD mutations. Those molecules displaying further improved fitness for the evolving feature, are referred to herein as super-LEADs.

Please replace the paragraph on page 7, lines 24-28, with the following amended paragraph:

Figure 5 depicts different levels of biological activity of a protein, designated Rep protein, super-LEADs obtained by ADM. Rep protein is [[is]] involved in replication of ~~Adeno-associated~~ adeno-associated virus (see, *e.g.*, copending U.S. application Serial No. 10/022,390, published as US-2003-0129203-A1). It was used to exemplify the ADM method.

Please replace the paragraph on page 12, lines 3-11, with the following amended paragraph:

As used herein, two dimensional (2D) rational mutagenesis scanning (also referred to herein as 2D-scanning) refers to the process provided herein in which two dimensions of a particular protein sequence are scanned: (1) in one dimension specific amino acid residues along the protein sequence for replacement with different amino acids are ~~identifed;~~identified; these are referred to as is-HIT target positions; and (2) in the second dimension the amino acid type for replacing a particular is-HIT target is selected, these amino acids are referred to as the replacing or replacement amino acid(s).

Please replace the paragraph on page 17, lines 12-22, with the following amended paragraph:

As used herein, the phrase "pseudo-wild type" amino acids in the context of single or multiple amino acid replacements, are those amino acids that are different from the native amino acid at a given amino acid position but can replace the native one at that position without introducing any measurable

change (typically a change less than 10%, 5% or 1%, depending upon the ~~activity)~~activity) in a particular protein activity. A population of sets of nucleic acid molecules encoding a collection of mutant molecules can be generated and phenotypically characterized such that proteins with amino acid sequences different from the native ones but that still elicit the same level and type of desired activity as the native protein can be produced.

Please replace the paragraph on page 18, line 25, to page 19, line 12, with the following amended paragraph:

As used herein, the Hill equation is a mathematical model that relates the concentration of a drug (*i.e.*, test compound or [[substa nce]] substance) to the response measured

$$y = \frac{y_{max}[D]^n}{[D]^n + [D_{50}]^n},$$

where y is the variable measured, such as a response or signal, y_{max} is the maximal response achievable, [D] is the molar concentration of a drug, $[D_{50}]$ is the concentration that produces a 50% maximal response to the drug, n is the slope parameter, which is 1 if the drug binds to a single site and with no cooperativity between or among sites. A Hill plot is $\log_{10}$ of the ratio of ligand-occupied receptor to free receptor vs. log [D] (M). The slope is n, where a slope of greater than 1 indicates cooperativity among binding sites, and a slope of less than 1 can indicate heterogeneity of binding. This general equation has been employed for assessing interactions in complex biological systems (see, published International PCT application No. WO 01/44809 based on PCT No. PCT/FR00/03503, see, also, the EXAMPLES).

Please replace the paragraph on page 20, lines 11-18, with the following amended paragraph:

As used herein, a population of sets of nucleic acid molecules encoding a collection of mutants refers to a collection of plasmids or other vehicles that carrying (encoding) the gene variants, such that individual plasmid or other vehicles carry individual gene variants. Each element of the collection (library) is physically separated from the others, individually set in an appropriate format, such [[asn]] as an addressable array, and is generated as a single product of an independent mutagenesis reaction. When a collection of proteins is contemplated, it will be so-stated.

Please replace the paragraph on page 28, line 28, to page 29, line 21, with the following amended paragraph:

As used herein, a support (also referred to as a matrix support, a matrix, an insoluble support or solid support) refers to any solid or semisolid or insoluble support to which a molecule of interest, typically a biological molecule, organic molecule or biospecific ligand is linked or contacted. Such materials include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but are not limited to: polystyrene, polycarbonate, polypropylene, nylon, glass, dextran, chitin, sand, pumice, agarose, polysaccharides, dendrimers, buckyballs, polyacryl-amide, silicon, rubber, and other materials used as supports for solid phase syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications. The matrix herein can be particulate or can be in the form of a continuous surface, such as a microtiter dish or well, a glass slide, a silicon chip, a nitrocellulose sheet, nylon mesh, or other such materials. When particulate, typically the particles have at least one dimension in the 5-10 mm range or smaller. Such particles, referred collectively herein as "beads," are often, but not necessarily, spherical. Such reference, however, does not constrain the geometry of the matrix, which may be any shape, including random shapes, needles, fibers, and elongated. Roughly spherical "beads," particularly microspheres that can be used in the liquid phase, also are

contemplated. The "beads" may include additional components, such as magnetic or paramagnetic particles (see, *e.g.*, ~~Dynabeads~~ DYNABEADS ([[Dynal]] DYNAL, Oslo, Norway)) for separation using magnets, as long as the additional components do not interfere with the methods and analyses herein.

Please replace the paragraph on page 36, lines 6-12, with the following amended paragraph:

The "pure random mutagenesis" approach would proceed by blinded random (stochastic) amino acid replacement at any place on the protein sequence, whether the protein 3-dimensional structure is known or not. The "restricted random mutagenesis" approach, however, is performed in the absence of knowledge about the 3-dimensional structure. Where [[where]] the 3-dimensional structure of the protein is known, this method joins and becomes a sort of "pure random mutagenesis" approach.

Please replace the paragraph on page 37, line 14, to page 38, line 3, with the following amended paragraph:

The 2D rational scanning methods provided herein still maintain the value of performing a "blinded" screening, that is observed in the other three approaches; although it is more conditioned by previous knowledge of amino acid properties, in the sense that it relies on a higher number of assumptions and hypotheses. This effect is partially countered by the fact that as many alternative is-HIT positions as possible, identified based on different criteria (helix-turn disruption, hydrophobicity, and other parameters), are covered. On the other hand, the number of different replacing amino acids is kept as large as reasonably possible, up to all the 20 amino acids (at each position), whenever appropriate. Despite [[of]] the restrictions introduced by the rational assumptions made in the choice of is-HIT target positions and of the replacing amino acids, because the selection of both is-HIT target positions and replacing amino acids is limited to a minimum (keeping the number of is-HIT as large as

possible) and the replacing amino acid type as broad as possible, the 2D-scanning method provided herein is extremely rich in its potential for exploring unexpected and innovative amino acid sequences, while at the same time, being highly efficient in terms of attrition rate between mutants generated and LEAD molecules obtained.[[.]] Given the number of different candidate LEAD protein molecules that are generated (e.g., a few thousands per collection), a high-throughput screening is typically necessary.

Please replace the paragraph on page 49, lines 9-31, with the following amended paragraph:

A population of sets of nucleic acid molecules encoding a collection of new super-LEAD mutant molecules is generated, tested and phenotypically characterized one-by-one in addressable arrays. [[super-LEAD]] Super-LEAD mutant molecules are such that each molecule contains a variable number and type of LEAD mutations. Those molecules displaying further improved fitness for the particular feature being evolved, are referred to as super-LEADs. Super-LEADs may be generated by other methods known to those of skill in the art and tested by the high throughput methods herein. For purposes herein a super-LEAD typically has activity with respect to the function or biological activity of interest that differs from the improved activity of a LEAD by a desired amount, such as at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200% or more from at least one of the LEAD mutants from which it is derived. In yet other embodiments, the change in activity is at least about 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times, 200 times, 300 times, 400 times, 500 times, 600 times, 700 times, 800 times, 900 times, 1000 times, or more greater than at least one of the LEAD molecules from which it is derived. As with LEADs, the change in the activity for super-LEADs is dependent upon the activity that is being "evolved."

The desired alteration, which can be either an increase or a reduction in activity, will depend upon the function or property of interest.

Please replace the paragraph on page 51, lines 1-20, with the following amended paragraph:

Another method for generation of ~~super-leads~~super-LEADs is multi-overlapped ~~primier~~primer extensions. This is a method for the rational evolution of proteins using oligonucleotide-mediated mutagenesis. This method is particularly useful for the rational combination of mutant LEADs to form super-LEADs (see FIG**14**). This method allows the simultaneous introduction of several mutations throughout a small protein or protein-region of known sequence (see, e.g., FIGS**13**A through D). Overlapping oligonucleotides of typically around 70 bases in length (since longer oligonucleotides LEAD to increased error) are designed from the DNA sequence (gene) encoding the mutant LEAD proteins in such a way that they overlap with each other on a region of typically around 20 bases. These overlapping oligonucleotides (including or not point mutations) act as both template and primers in a first step of PCR (using a proofreading polymerase, e.g., Pfu DNA polymerase, to avoid unexpected mutations) to create small amounts of full-length gene. The full-length gene resulting from the first PCR then is selectively amplified in a second step of PCR using flanking primers, each one tagged with a restriction site in order to facilitate subsequent cloning. One multi-overlapped extension process yields a full-length (multi-mutated) nucleic acid molecule encoding a candidate super-LEADs protein having multiple mutations therein derived from LEAD mutant proteins.

Please replace the paragraph on page 52, lines 8-12 with the following amended paragraph:

The 2D methods provided herein are used to alter activity or physical or chemical property of a target polypeptide. Any characteristic (physical,

chemical property or activity) can be modified. The protein is selected and the property identified. A suitable assay or method for identifying proteins with the ~~characterisitic.~~ characteristic.

Please replace the paragraph on page 54, lines 4-20, with the following amended paragraph:

(2) Since protease mixtures in the body are quite complex in composition, almost all the residues in a selected protein sequence ~~potentialy~~potentially could be targeted for proteolysis (FIG6A). Nevertheless, proteins form specific tri-dimensional structures where residues are more or less exposed to the environment and protease action. It can be assumed that those residues constituting the core of a protein are inaccessible to proteases, while those more "exposed" to the environment are better targets for proteases. The probability for every specific amino acid to be "exposed" and accessible to proteases can be taken into account to reduce the number of is-HITs. Consequently, the methods herein consider the analysis with respect to solvent "exposure" or "accessibility" for each individual amino acid in the protein sequence. Solvent accessibility of residues can alternatively be estimated, regardless of any previous knowledge of specific protein structural data, by using an algorithm derived from empirical amino acid probabilities of accessibility, which is expressed in the following equation (Boger *et al.*, *Reports of the Sixth International Congress in Immunology*, p. 250, 1986):

Please replace the paragraph on page 56, lines 9-21, with the following amended paragraph:

IFNα-2b is used for a variety of applications. Typically it is used for treatment of type B and C chronic hepatitis. Additional indications include, but are not limited to, melanomas, herpes infections, ~~Kaposi~~ Karposi sarcomas and some leukemia and lymphoma cases. Patients receiving interferon are subject to frequent repeat applications of the drug. Since such frequent injections

generate uncomfortable physiological as well as undesirable psychological reactions in patients, increasing the half-life of interferons and thus decreasing the necessary frequency of interferon injections, would be extremely useful to the medical community. For example, after injection of native human IFNα-2b injection in mice, as a model system, its presence can be detected in the serum between 3 and 10 hours with a half-life of only around 4 hours. The IFNα-2b completely disappears to undetectable levels by 18-24 hours after injection.

Please replace the paragraph on page 63, line 2, to page 64, line 2, with the following amended paragraph:

Two methodologies were used to address the improvements described above: (a) 2D-scanning methods were used to identify aminoacid changes that lead to improvement in protease resistance and to improvement in conformational stability, and (b) 3D-scanning, which employs structural homology methods (see, copending U.S. application Serial No. ~~attorney dkt. no.37851-922~~ 10/658,834, filed the same day herewith, based upon U.S. provisional application Serial Nos. 60/457,135 and 60/409,898) methods also were used to identify ~~aminoacid~~ amino acid changes that lead to improvement in protease resistance.

Please replace the paragraph on page 65, lines 5-17, with the following amended paragraph:

For the improvement of resistance to proteases, by 3D-scanning (structural homology):

1) Identifying some or all possible target sites (is-HITS) on the protein sequence that display an acceptable degree of structural homology around the ~~aminoacid~~ amino acid positions mutated in the LEAD molecules previously obtained for IFNα using 2D-scanning, and that are susceptible to digestion by one or more specific proteases; and

2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at the same time, keeping the biological activity of the protein unchanged (these replacing amino acids are the candidate LEADs).

Please replace the paragraph on page 81, lines 1-18, with the following amended paragraph:

The rational mutagenesis methods provided herein also can be used to evolve proteins that are contained in agronomic consumables, crops or foodstuff, such that these proteins display either decreased or abolished secondary effects (such as toxic or allergenic effects) on the consumer. For example, toxic or allergenic effects are attributable to a lack of (or incomplete) digestion of particular proteins in the gut. Thus, it would be useful to increase digestibility of the proteins concerned, while preserving their biological activity. For this purpose, a similar approach to the methods provided herein for increasing protein stability (e.g., see IFNα-2b mutants herein) can be used. Most allergens are resistant to gastric acid and to digestive proteases (Fuchs *et al.*, *Food Technology*, 50:83-88, 1996; Astwood *et al.*, *Nature Biotechnology*, 14:1269-1273, 1996), whereas common plant proteins are not. Since agronomic consumables, crops or foodstuff are typically for oral consumption, proteases of the luminal gastrointestinal tract, such as pepsin, trypsin and ~~chimiotrypsin~~ chymotrypsin (Woodley, *Crit. Rev. Ther. Drug.*, 11:61-95, 1994; Bernkop-Schnürch, *J. Control. Release*, 52:1-16, 1998), are included in the list of proteases by which the evolving protein is rendered digestible.

Please replace the paragraph on page 96, line 18, to page 97, line 14, with the following amended paragraph:

Accordingly, provided herein are IFNα-2b mutant proteins that contain one or more pseudo-wild type mutations at amino acid positions of IFNα-2b

corresponding to SEQ ID NO:1, amino acid residues: 9, 10, 17, 20, 24, 25, 35, 37, 41, 52, 54, 56, 57, 58, 60, 63, 64, 65, 76, 89, and 90. The mutations can be either one or more of insertions, deletions and/or replacements of the native amino acid residue(s). In one embodiment, the psuedo-wild type replacements are mutations with alanine at each position. In another embodiment, the pseudo-wild type replacements are one or more mutations in SEQ ID NO:1 corresponding to:

P by A at position 4, Q by A at position 5 ,
T by A at position 6, L by A at position 9,
[[LG]] G by A at position 10, L by A at position 17,
Q by A at position 20, I by A at position 24,
S by A at position 25, D by A at position 35,
G by A at position 37, G by A at position 39,
E by A at position 41, E by A at position 42,
E by A at position 51, T by A at position 52,
P by A at position 54, V by A at position 55,
L by A at position 56, H by A at position 57,
E by A at position 58, I by A at position 60,
I by A at position 63, F by A at position 64,
N by A at position 65, W by A at position 76,
D by A at position 77, E by A at position 78,
L by A at position 81, Y by A at position 85,
Y by A at position 89, Q by A at position 90,
G by A at position 104, L by A at position 110,
S by A at position 115 and E by A at position 146.

Please replace the paragraph on page 98, line 29, to page 99, line 26, with the following amended paragraph:

3-dimensionally structurally equivalent or similar amino acid positions that are located on two or more different protein sequences that share a certain

degree of structural homology, have comparable functional tasks (activities and phenotypes). These two amino acids that occupy substantially equivalent 3-dimensional structural space within their respective proteins then can be said to be "structurally similar" or "structurally related" to each other, even if their precise positions on the amino acid sequences, when these sequences are aligned, do not match with each other. The two amino acids also are said to occupy "structurally homologous loci." "Structural homology" does not take into account the underlying amino acid sequence and solely compares 3-dimensional structures of proteins. Thus, two proteins can be said to have some degree of structural homology whenever they share conformational regions or domains showing comparable structures or shapes with 3-dimensional overlapping in space. Two proteins can be said to have a higher degree of structural homology whenever they share a higher amount of conformational regions or domains showing comparable structures or shapes with 3-dimensional overlapping in space. Amino [[acids]] acid positions on one or more proteins that are "structurally homologous" can be relatively far way from each other in the protein sequences, when these sequences are aligned following the rules of primary sequence homology. Thus, when two or more protein backbones are determined to be structurally homologous, the amino acid residues that are coincident upon three-dimensional structural superposition are referred to as "structurally similar" or "structurally related" amino acid residues in structurally homologous proteins (also referred to as "structurally homologous loci"). Structurally similar amino acid residues are located in substantially equivalent spatial positions in structurally homologous proteins.

Please replace the paragraphs on page 99, line 27 to page 100, line 18, with the following amended paragraphs:

For example, for proteins of average size (approximately 180 residues), two structures with a similar fold will usually display [[rms]] RMS deviations not exceeding 3 to 4 angstroms. For example, structurally similar or structurally

related amino acid residues can have backbone positions less than 3.5, 3.0, 2.5, 2.0, 1.7 or 1.5 angstrom from each other upon protein superposition. RMS deviation calculations and protein superposition can be carried out using any of a number of methods known in the art. For example, protein superposition and RMS deviation calculations can be carried out using all peptide backbone atoms (e.g., N, C, C(C=O), O and CA (when present)). As another example, protein superposition can be carried out using just one or any combination of peptide backbone atoms, such as, for example, N, C, C(C=O), O and CA (when present). In addition, one skilled in the art will recognize that protein superposition and RMS deviation calculations generally can be performed on only a subset of the entire protein structure. For example, if the protein superposition is carried out using one protein that has many more amino acid residues than another protein, protein superposition can be carried out on the subset (e.g., a domain) of the larger protein that adopts a structure similar to the smaller protein. Similarly, only portions of other proteins can be suitable for superimposition. For example, if the position of the C-terminal residues from two structurally homologous proteins differ significantly, the C-terminal residues can be omitted from the structural superposition or RMS deviation calculations.

Please replace the paragraphs on page 107, lines 12-25, with the following amended paragraphs:

The pSSV9 CMV 0.3 pA was cut by *Pvu*II and religated (this step gets rid of the ITR functions), prior to the introduction of a new *Eco*RI restriction site by Quickchange mutagenesis ~~(Stratagene)~~ (STRATAGENE). The oligonucleotides primers were:

EcoRI forward primer 5'-GCCTGTATGATTTATTGGATGTTGGAATTCC-CTGATGCGGTATTTTCTCCTTACG-3' (SEQ ID NO: 182)

EcoRI reverse primer 5'-CGTAAGGAGAAAATACCGCATCAGGGAATT-CCAACATCCAATAAATCATACAGGC-3' (SEQ ID NO: 183).

The construct sequence was confirmed by using the following oligonucleotides:

Seq ClaI forward primer: 5'-CTGATTATCAACCGGGGTACATATGATTGAC-ATGC-3' (SEQ ID NO: 184)

Seq XmnI reverse primer: 5'-TACGGGATAATACCGCGCCACATAGCAGAA-C-3' (SEQ ID NO: 185).

Please replace the paragraphs on page 108, line 30, to 109 line 7, with the following amended paragraphs:

~~BL21-CodonPlus(DE3)-RP®~~ BL21-CODONPLUS(DE3)-RP competent Escherichia coli cells are derived from ~~Stratagene's~~ STRATAGENE's high-performance BL21-Gold competent cells. These cells enable efficient high-level expression of heterologous proteins in *E. coli*. Efficient production of heterologous proteins in E. coli is frequently limited by the rarity, in E.coli, of certain tRNAs that are abundant in the organisms from which the heterologous proteins are derived. Availability of tRNAs allows high-level expression of many heterologous recombinant genes in ~~BL21-Codon Plus~~ BL21-CODONPLUS cells that are poorly expressed in conventional BL21 strains.~~BL21-CodonPLus(DE3)-RP~~ BL21-CODONPLUS(DE3)-RP cells contain a ColE1-compatible, pACYC-based plasmid containing extra copies of the argU and proL tRNA genes.

Please replace the paragraphs on page 109, lines 9-23, with the following amended paragraphs:

To express IFN α-2b in *E.coli* cDNA encoding the mature form of IFN-2 α-2b was finally cloned into the plasmid pET-11 ~~(Novagen)~~ (NOVAGEN). Briefly, this cDNA fragment was amplified by PCR using the primers SEQ ID [[Nos.]] NOS: 208 and 209, respectively:

FOR-IFNA-5' AACATATGTGTGATCTGCCTCAAACCCACAGCCTGGGTAGC 3'

REV-IFNA-5' AAGGATCCTCATTCCTTACTTCTTAAACTTTCTTGCAAGTTTGTTG3',

from pSSV9-EcoRI-IFN α-2b (see above), which contains full-length IFN-2 alpha cDNA as a matrix, using ~~Herculase~~ HERCULASE DNA-polymerase ~~(Stratagene)~~ (STRATAGENE). The PCR fragment was subcloned into pTOPO-TA vector ~~(Invitrogen)~~ (INVITROGEN) yielding pTOPO-IFN α-2b. The sequence was verified by sequencing. pET11 IFN α-2b was prepared by insertion of the *NdeI-Bam HI* (Biolabs) fragment from pTOPO-IFN α-2b into the *NdeI-Bam HI* sites of pET 11. The DNA sequence of the resulting pET 11-IFN α-2b construct was verified by sequencing and the plasmid was used for IFN α-2b expression in *E.coli*.

Please replace the paragraphs on page 109, line 26, to 110 line 12, with the following amended paragraphs:

Lead mutants of Interferon alpha were first generated in the pSSV9-IFNa-EcoRI plasmid. With the only exception of E159H and E159Q, all mutants were amplified using the primers below. Primers contained NdeI (in Forward) and BamHI (in Reverse) restriction sites:

FOR-IFNA-5' AAC ATA TGT GTG ATC TGC CTC AAA CCC ACA GCC TGG GTA GC 3' SEQ ID No. 210; and

REV-IFNA-5' AAG GAT CCT CAT TCC TTA CTT CTT AAA CTT TCT TGC AAG TTT GTT G 3' SEQ ID No. 211.

Mutants E159H and E159Q were amplified using the following primers on reverse side (primer forward was the same than described above):
REV-IFNA-E159H-5' AAG GAT CCT CAT TCC TTA CTT CTT AAA CTG TGT TGC AAG TTT GTT G 3' SEQ ID No. 500.
REV-IFNA-E159Q-5' AAG GAT CCT CAT TCC TTA CTT CTT AAA CTC TGT TGC AAG TTT GTT G 3' SEQ ID No. 501.
Mutants were amplified with Pfu Turbo Polymerase ~~(Stratagene)~~ STRATAGENE according to the manufacturers's instructions. PCR products were cloned into pTOPO plasmid (Zero Blunt TOPO PCR cloning kit, ~~Invitrogen).~~ INVITROGEN). The presence of the desired mutations was checked by automatic sequencing. The NdeI + BamHI fragment of the pTOPO-IFNa positive clones was then cloned into NdeI + BamHI sites of the pET11 plasmid.

Please replace the paragraphs on page 110, line 15, to page 111 line 26, with the following amended paragraphs:

A series of mutagenic primers was designed to generate the appropriate site-specific mutations in the IFNα-2b cDNA. Mutagenesis reactions were performed with the Chameleon® mutagenesis kit ~~(Stratagene)~~ (STRATAGENE) using pNB-AAV-IFNα-2b as the template. Each individual mutagenesis reaction was designed to generate one single mutant protein. Each individual

mutagenesis reaction contains one and only one mutagenic primer. For each reaction, 25 pmoles of each (phosphorylated) mutagenic primer were mixed with 0.25 pmoles of template, 25 pmoles of selection primer (introducing a new restriction site), and 2 μl of 10X mutagenesis buffer (100 mM Tris-acetate pH 7.5; 100 mM MgOAc; 500 mM KOAc pH 7.5) into each well of 96 well-plates. To allow DNA annealing, PCR plates were incubated at 98 °C during 5 min and immediately placed 5 min on ice, before incubating at room temperature during 30 min. Elongation and ligation reactions were allowed by addition of 7 μl of nucleotide mix (2.86 mM each nucleotide; 1.43 X mutagenesis buffer) and 3 μl of a freshly prepared enzyme mixture of dilution buffer (20 mM Tris HCl pH7.5; 10 mM KCl; 10 mM β-mercaptoethanol; 1 mM DTT; 0.1 mM EDTA; 50 % glycerol), native T7 DNA polymerase (0.025 U/μl), and T4 DNA ligase (1 U/μl) in a ratio of 1:10, respectively. Reactions were incubated at 37 °C for 1 h before inactivation of T4 DNA ligase at 72 °C during 15 min. In order to eliminate the parental plasmid, 30 μl of a mixture containing 1X enzyme buffer and 10 U of restriction enzyme was added to the mutagenic reactions followed by incubation at 37 °C for at least 3 hours. Next, 90 μl aliquots of XL*mutS* competent cells ~~(Stratagene)~~ (STRATAGENE) containing 25 mM β-mercaptoethanol were place in ice-chilled deep-well plates. Then, plates were incubated on ice for 10 min with gentle vortex every 2 min. Transformation of competent cells was performed by adding aliquots of the restriction reactions (1/10 of reaction volume) and incubating on ice for 30 min. A heat pulse was performed in a 42 °C water bath for 45 s, followed by incubation on ice for 2 minutes. Preheated SOC medium (0.45 ml) was added to each well and plates were incubated at 37 °C for 1 h with shaking. In order to enrich for mutated plasmids, 1 ml of 2 X YT broth medium supplemented with 100 μg/ml ampicillin was added to each transformation mixture followed by overnight incubation at 37 °C with shaking. Plasmid DNA isolation was performed by alkaline lysis using ~~Nucleospin~~ NUCLEOSPIN Multi-96 Plus Plasmid Kit (Macherey-Nagel) according to the manufacturer's instructions. Selection of mutated plasmids was performed by

digesting 500 μg of plasmid preparation with 10 U of selection endonuclease in an overnight incubation at 37 °C. A fraction of the digested reactions (1/10 of the total volume) was transformed into 40 μl of ~~Epicurian~~ EPICURIAN coli XL1-Blue competent cells ~~(Stratagene)~~ (STRATAGENE) supplemented with 25 mM β-mercaptoethanol.

Please replace the paragraph on page 112, lines 7-18, with the following amended paragraph:

IFNα-2b mutants were produced in 293 human embryo kidney (HEK) cells (obtained from ATCC), using ~~Dubelcco's~~ Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g/L; ~~Gibco-BRL~~ GIBCO-BRL) and fetal bovine serum (10%, ~~Hyclone~~ HYCLONE). Cells were transiently transfected with the plasmids encoding the IFNα-2b mutants as follows: 0.6 x 10^5 cells were seeded into 6 well-plates and grown for 36 h before transfection. Confluent cells at about 70%, were supplemented with 2.5 μg of plasmid (IFNα-2b mutants) and [[10 Mm]] 10 mM poly-ethylene-imine (25 KDa PEI, ~~Sigma-Aldrich).~~ SIGMA-ALDRICH). After gently shaking, cells were incubated for 16 h. Then, the culture medium was changed with 1 ml of fresh medium supplemented with 1% of serum. IFNα-2b was measured on culture supernatants obtained 40 h after transfection and stored in aliquots at -80 °C until use.

Please replace the paragraph on page 113, lines 1-5, with the following amended paragraph:

A volume of 200 ml of culture medium (LB/Ampicillin/ Chloramphenicol) was inoculated with 5 ml of pre-culture BL21-pCodon+-pET-IFN α-2b [[muta]] mutant overnight at 37 °C with constant shaking (225 rpm). The production of IFN α-2b was induced by the addition of 50 μl of 2M IPTG at $DO_{600nm} \sim 0.6$.

Please replace the paragraph on page 113, lines 16-23, with the following amended paragraph:

Pellets containing the inclusion bodies were suspended in 10 ml of buffer and sonicated (80 watts) on ice, 1 second "on", 1 second "off" for a total of 4 min. Suspensions were then centrifuged (4°C, 10000 g, 15 min), and supernatants were recovered. Pellets were resuspended in 10 ml of buffer for a new sonication/centrifugation cycle. ~~Triton X-100~~ TRITON X-100 was then eliminated by two additional cycles of sonication/centrifugation with buffer. Pellets containing the inclusion bodies were recovered and dissolved. The washed supernatants were stored at 4°C.

Please replace the paragraph on page 113, line 30, to page 114, line 5, with the following amended paragraph:

Samples contained 1 mg of protein at 0.3 mg/ml (5 ml in total) in buffer. The GdnHCl [[(Hydrochloride Guanidium)]] guanidinium hydrochloride present in the samples was eliminated by dialysis (minimum membrane cut = 10 kDa) overnight at 4°C against buffer (1litre) (final concentration of GdnHCl : 43 Mm). Next, samples were further dialysed against 1litre of buffer during 2:30h. This step was repeated two additional times. After dialysis, very little precipitate was visible.

Please replace the paragraph on page 11, lines 4-16, with the following amended paragraph:

D. Screening and *in vitro* ~~charaterization~~characterization of IFN α-2b mutants

Two activities were measured directly on IFN samples: antiviral and antiproliferation activities. Dose (concentration) - response (activity) experiments for antiviral or antiproliferation activity permitted calculation of the 'potency' for antiviral and antiproliferation activities, respectively. Antiviral and antiproliferation activities also were measured after incubation with proteolytic samples, such as specific proteases, mixtures of selected proteases, human serum or human blood. Assessment of activity following incubation with proteolytic samples allowed to determine the residual (antiviral or

antiproliferation) activity and the respective kinetics of half-life upon exposure to proteases.

Please replace the paragraph on page 114, line 27, to page 115, line 20, with the following amended paragraph:

Confluent cells were trypsinized and plated at density 2 x 10^4 cells/well in DMEM 5% SVF medium (Day 0). Cells were incubated with IFN α-2b (at a concentration of 500 U/ml) to get 500 pg/ml and 150 pg/well (100 μl of IFN solution), during 24 h at 37 °C prior to be challenged with EMCV (1/1000 dilution; MOI 100). After an incubation of 16 h, when virus–induced CPE was near maximum in untreated cells, the number of EMCV particles in each well was determined by RT-PCR quantification of EMCV mRNA, using lysates of infected cells. RNA from cell extracts was purified after a DNAse/proteinase K treatment ~~(Applied Biosystems)~~ (APPLIED BIOSYSTEMS). The CPE was evaluated using both Uptibleu (Interchim) and MTS ~~(Promega)~~ (PROMEGA) methods, which are based on detecting bio-reductions produced by the metabolic activity of cells in a ~~flourometric~~ fluorimetric and colorimetric manner, respectively. In order to produce a standard curve for EMCV quantification, a 22 bp DNA fragment of the capsid protein-cDNA was amplified by PCR and cloned into pTOPO-TA vector ~~(Invitrogen)~~ (INVITROGEN). Next, RT-PCR quantification of known amounts of pTOPO-TA-EMCV capsid gene was performed using the One-step RT-PCR kit ~~(Applied Biosystems)~~ (APPLIED BIOSYSTEMS) and the following EMCV-related (cloning) oligonucleotides and probe:

EMCV forward primer 5'-CCCCTACATTGAGGCATCCA-3' (SEQ ID NO: 193)

EMCV reverse primer 5'-CAGGAGCAGGACAAGGTCACT-3' (SEQ ID NO: 194)

EMCV probe 5'-(FAM)CAGCCGTCAAGACCCAACCGCT(TAMR A)-3' (SEQ ID NO: 195).

Please replace the paragraph on page 115, line 29, to page 116, line 17, with the following amended paragraph:

Two-fold serial dilutions of interferon samples were made with MEM complete media into 96-Deep-Well plates with final concentration ranging from 1600 to 0.6 pg/ml. The medium was aspirated from each well and 100 μl of interferon dilutions were added to Hela cells. Each interferon sample dilution was assessed in triplicate. The two last rows of the plates were filled with 100 μl of medium without interferon dilution samples in order to serve as controls for cells with and without virus. After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well except for the cell control row. Plates were returned to the CO_2 incubator for 48 hours. Then, the medium was aspirated and the cells were stained for 1 hour with 100 μl of Blue staining ~~solutio~~ solution to determine the proportion of intact cells. Plates were washed in a distilled water bath. The cell bound dye was extracted using 100 μl of ethylene-glycol mono-ethyl-ether ~~(Sigma)~~ (SIGMA). The absorbance of the dye was measured using an Elisa plate reader ~~(Spectramax)~~ (SPECTRAMAX). The antiviral activity of IFN α-2b samples (expressed as number of IU/mg of proteins) was determined as the concentration needed for 50% protection of the cells against EMC virus-induced cytopathic effects. For proteolysis experiments, each point of for the kinetic measurements was assessed at 500 and 166 pg/ml in triplicate.

Please replace the paragraph on page 116, lines 19-25, with the following amended paragraph:

Anti-proliferative activity of interferonα-2b was determined by the capacity of the cytokine to inhibit proliferation of Daudi cells. Daudi cells ($1x10^4$ cells) were seeded in flat-bottomed 96-well plates containing 50μl/well of RPMI 1640 medium supplemented with 10% SVF, 1X ~~glutamin~~ glutamine and 1ml of gentamicin. No cell was added to the last row ("H" row) of the flat-bottomed 96-well plates in order to evaluate background absorbance of culture medium.

Please replace the paragraph on page 117, lines 4-9, with the following amended paragraph:

After 72 hours of growth, 20 µl of Cell titer 96 Aqueous one solution reagent ~~(Promega)~~ (PROMEGA) was added to each well and incubated 1H30 at 37°C in an atmosphere of 5% CO_2. To measure the amount of colored soluble formazan produced by cellular reduction of the MTS, the absorbance of the dye was measured using an Elisa plate reader ~~(spectramax)~~ (SPECTRAMAX) at 490nm.

Please replace the paragraph on page 117, lines 17-28, with the following amended paragraph:

Mutants were treated with proteases in order to identify resistant molecules. The resistance of the mutant IFN α-2b molecules compared to wild-type IFN α-2b against enzymatic cleavage (30 min, 25 °C) by a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, [[Sigma]] SIGMA): α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and trypsin) was determined. At the end of the incubation time, 10 µl of anti-proteases complete, mini EDTA free, [[Roche]] ROCHE (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity. Treated samples were then used to determine residual antiviral or antiproliferation activities.

Please replace the paragraph on page 117, line 30, to page 118, line 10, with the following amended paragraph:

The percent of residual IFN α-2b activity over time of exposure to proteases was evaluated by a kinetic study using either (a) 15 pg of chymotrypsin (10%wt/wt), (b) a lysate of human blood at dilution 1/100, (c) 1.5 pg of protease mixture, or (d) human serum. Incubation times were: 0 h, 0.5 h, 1 h, 4 h, 8 h, 16 h, 24 h and 48 h. Briefly, 20 µl of each proteolytic sample

(proteases, serum, [[bnlood]] blood) was added to 100 μl of IFN α-2b at 1500 pg/ml (500U/ml) and incubated for variable times, as indicated. At the appropriate time points, 10 μl of anti-proteases mixture, mini EDTA free, [[Roche]] ROCHE (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.

Please replace the paragraph on page 118, line 22, to page 119, line 3, with the following amended paragraph:

IFNα –2b mutants selected on the basis of their overall performance in vitro, were tested for pharmacokinetics in mice in order to have an indication of their half-life in blood in vivo. Mice were treated by subcutaneous (SC) injection with ~~alieuots~~ aliquots of each of a number of selected lead mutants. Blood was collected at increasing time points between 0.5 and 48 [[hs]] hours after injection. ~~Inmediatedly~~ Immediately after collection, 20 ml of anti-protease solution were added to each blood sample. Serum was obtained for further ~~analysis.Residual~~ analysis. Residual IFN-α activity in blood was determined using the tests described in the precedent sections for in vitro characterization. Wild-type IFN α (that had been produced in bacteria under comparable conditions as the lead mutants) as well as a pegylated derivative of IFN α, ~~Pegasys (Roche)~~ PEGASYS (ROCHE), also were tested for pharmacokinetics in the same experiments.

Please replace the paragraph on page 132, lines 3-10, with the following amended paragraph:

The pSSV9 CMV 0.3 pA was cut by *Pvu*II and religated (this step gets rid of the ITR functions), prior to the introduction of a new *Eco*RI restriction site by ~~Quickchange~~ QUICKCHANGE mutagenesis ~~(Stratagene)~~ (STRATAGENE). The oligonucleotides sequences used, follow:

EcoRI forward primer: 5'-GCCTGTATGATTTATTGGATGTTGGAATTCC-CTGATGCGGTATTTTCTCCTTACG-3' (SEQ ID NO: 182)

EcoRI reverse prime: 5'-CGTAAGGAGAAAATACCGCATCAGGGAATT-CCAACATCCAATAAATCATACAGGC-3' (SEQ ID NO: 183).

Please replace the paragraphs on page 132, line 27, to page 133, line 15, with the following amended paragraphs:

The verified IFN β-encoding cDNA first was cloned into the pTOPO-TA vector ~~(Invitrogen)~~ (INVITROGEN). After checking of the cDNA sequence by automatic DNA sequencing, the *Hin*dIII-*Xba*I fragment containing the IFN cDNA was subcloned into the corresponding sites of pSSV9-2EcoRI, leading to the construct pAAV-EcoRI-IFNbeta (pNB-AAV-IFN beta). Finally the fragment Pvu II of plasmid pNB-AAV-IFN beta was subcloned in PvuII site of pUC 18 leading the final construct pUC-CMVIFNbetapA called pNAUT-IFNbeta.

Production and normalization of IFNβ in mammalian cells

IFN β was produced in CHO Chinese Hamster Ovarian cells (obtained from ATCC), using ~~Dubelcco's~~ Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g/L; ~~Gibco BRL~~ GIBCO-BRL) and fetal bovine serum (5 %, ~~Hyclone~~ HYCLONE). Cells were transiently transfected as follows: 0.6 x 10^5 cells were seeded into 6 well plates and grown for 24 h before transfection. Confluent cells at about 70%, were supplemented with 1.0 μg of plasmid (from the library of IFN β mutants) by lipofectamine plus reagent ~~(Invitrogen)~~ (INVITROGEN). After gently shaking, cells were incubated for 24 h with 1 ml of culture medium supplemented with 1 % of serum. IFN β was obtained from culture supernatants 24 h after transfection and stored in aliquots at –80 °C until use.

Please replace the paragraphs on page 133, line 22, to page 134, line 2, with the following amended paragraphs:

Screening and in vitro ~~charaterization~~ characterization of IFN β mutants

Two activities were measured directly on IFN samples: antiviral and antiproliferation activities. Dose (concentration) - response (activity) experiments for antiviral or antiproliferation activity allowed for the calculation of the 'potency' for antiviral and antiproliferation activities, respectively. Antiviral and antiproliferation activities also were measured after incubation with proteolytic samples such as specific proteases, mixtures of selected proteases, human serum or human blood. Assessment of activity following incubation with proteolytic samples allowed to determine the residual (antiviral or antiproliferation) activity [[an.d]] and the respective kinetics of half-life upon exposure to proteases.

Please replace the paragraphs on page 134, lines 3-17, with the following amended paragraphs:

Antiviral activity - measured by Cytopathic Effects (CPE)

Antiviral activity of IFN β was determined by the capacity of the cytokine to protect Hela cells against EMC (mouse encephalomyocarditis) virus-induced cytopathic effects. The day before, Hela cells ($2x10^5$ cells/ml) were seeded in flat-bottomed 96-well plates containing 100 μl/well of Dulbecco's MEM-Glutamaxl-sodium pyruvate medium supplemented with 5% SVF and 0.2% of gentamicin. Cells were growth at 37°C in an atmosphere of 5% CO_2 for 24 hours. Two-fold serial dilutions of interferon samples were made with MEM complete media into 96-Deep-Well plates with final concentration ranging from 1600 to 0.6 pg/ml. The medium was aspirated from each well and 100 μl of interferon dilutions were added to Hela cells. Each interferon sample dilution was assessed in triplicate. The two last rows of the plates were filled with 100 μl of medium without interferon dilution samples in order to serve as controls for cells with and without virus.

Please replace the paragraphs on page 134, lines 18-30, with the following amended paragraphs:

After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well, except for the cell control row. Plates were returned to the CO_2 incubator for 48 hours. Then, the medium was aspirated and the cells were stained for 1 hour with 100 μl of Blue staining ~~solutio~~ solution to determine the proportion of intact cells. Plates were washed in a distilled water bath. The cell bound dye was extracted using 100 μl of ethylene-glycol mono-ethyl-ether [[(Sigma)]] (SIGMA). The absorbance of the dye was measured using an Elisa plate reader ~~(Spectramax)~~ (SPECTRAMAX). The antiviral activity of IFN β samples (expressed as number of IU/mg of proteins) was determined as the concentration needed for 50% protection of the cells against EMC virus-induced cytopathic effects. For proteolysis experiments, each point of the kinetic was assessed at 800 and 400 pg/ml in triplicate.

Please replace the paragraph on page 135, lines 17-22, with the following amended paragraph:

After 72 hours of growth, 20 μl of Cell titer 96 Aqueous one solution reagent ~~(Promega)~~ (PROMEGA) was added to each well and incubated 1H30 at 37°C in an atmosphere of 5% CO_2. To measure the amount of colored soluble formazan produced by cellular reduction of the MTS, the absorbance of the dye was measured using an Elisa plate reader ~~(spectramax)~~ (SPECTRAMAX) at 490nm.

Please replace the paragraph on page 135, line 30, to page 136, line 10, with the following amended paragraph:

Mutants were treated with proteases in order to identify resistant molecules. The resistance of the mutant ~~IFN βmolecules~~ IFN β molecules compared to wild-type IFN β against enzymatic cleavage (120 min, 25 °C) by a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, [[Sigma]] SIGMA): α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and

trypsin) was determined. At the end of the incubation time, 10 μl of anti-proteases complete, mini EDTA free, [[Roche]] ROCHE (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity. Treated samples were then used to determine residual antiviral or antiproliferation activities.

Please replace the paragraph on page 136, lines 11-22, with the following amended paragraph:

Protease resistance - Kinetic analysis

The percent of residual IFN β activity over time of exposure to proteases was evaluated by a kinetic study using 1.5 pg of protease mixture. Incubation times were: 0 h, 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h. Briefly, 20 μl of each proteolytic sample (proteases, serum, [[bnlood]] blood) was added to 100 μl of IFN β at 400 and 800 pg/ml and incubated for variable times, as indicated. At the appropriate time points, 10 μl of anti-proteases mixture, mini EDTA free, [[Roche]] ROCHE (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.